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Oxygen-evolving extrinsic proteins (PsbO,P,Q,R): Bioinformatic and functional analysis

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Abstract

The water-splitting and oxygen-evolving (OE) reaction is carried out by a large multisubunit protein complex, Photosystem II (PSII), that has two distinct regions: a membrane intrinsic-region that includes most of the PSII subunits and a lumenal extrinsic-region that is in close association to the manganese catalytic center. The recently determined PSII 3D structures from cyanobacteria provide a considerable amount of new knowledge about the OE architecture (K.N. Ferreira, T.M. Iverson, K. Maghlaoui, J. Barber, S. Iwata, Architecture of the photosynthetic oxygen-evolving center, Science 303 (2004) 1831–1838; B. Loll, J. Kern, W. Saenger, A. Zouni, J. Biesiadka, Towards complete cofactor arrangement in the 3.0 A resolution structure of photosystem II, Nature 438 (2005) 1040–1044). Most of the intrinsic core PSII polypeptides have been well conserved through evolution from ancient cyanobacteria to modern plants, keeping the essence of PSII light driven reactions from prokaryotes to eukaryotes; but what is striking is the large number of changes that have occurred in the oxygen-evolving extrinsic proteins (OEEp) associated to PSII lumenal side. For unknown reasons plant PSII has required the "invention" of three OEEps: PsbP (23 kDa), PsbQ (16 kDa) and PsbR (10 kDa); associated to the ubiquitous OEEp PsbO (33 kDa). This set of proteins seems to be required in plants for the full activity and stability of the OE center *in vivo*, but their specific function is not clear. In this paper, bioinformatics and functional data show that the OEEps present in plants and green algae are very distinct from their prokaryotic counterparts. Moreover, clear differences are found for PsbQ from higher plants and green algae; and a relationship has been found between PsbR and the Mn cluster.

Keywords: Oxygen-evolving complex; Photosystem II complex; PsbO; PsbP; PsbQ; PsbR; Protein structure; Protein evolution; Bioinformatics

1. Introduction

The appearance of the oxygen-evolving (OE) bioreaction that liberates molecular oxygen to the atmosphere was an early event during the course of evolution and occurred in ancient cyanobacteria-like microorganisms. This fundamental reaction, powered by light energy (solar light) and present in all known oxyphototrophs, uses water (H_2O) as substrate and releases molecular di-oxygen (O_2) as by-product. The water splitting and oxygen release occurs in the catalytic center of Photosystem II (PSII), that is often termed the oxygen-evolving center (OEC) and includes a manganese-calcium cluster (4:1 Mn:Ca) as main catalytic cofactors. PSII is a large protein complex that has more than twenty different polypeptidic subunits, a major part inserted in the photosynthetic membranes as intrinsic-proteins (Ip) and another smaller set associated to the lumenal side of the complex as extrinsic-proteins (Ep). In addition to these polypeptides, PSII has many different cofactors: pigments (chlorophylls and carotenoids), metals (Mn, Ca, Fe), quinones. Recent 3D X-ray structures of cyanobacterial PSII have been obtained at 3.5 and 3.0 Å resolution [1,2], assigning most subunits and cofactors and providing a model of the OEC. However, no such high-resolution information is available for PSII of plants and green algae with the main differences being in the region corresponding to the lumenal side of the complex that includes the OE extrinsic proteins (OEEps) [3]. Here we use bioinformatics and functional data to explore the special nature of the OEEps of plants and green algae.

2. Methods

Isolation of PSII enriched membranes from spinach leaves was achieved as described in [4]. Calcium chloride washing of the PSII enriched membranes was conducted as described in [5]. SDS/PAGE of proteins in the membranes was performed according to the procedure described in [6]. Determination of Mn

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concentration in the PSII enriched membranes preparations was done using atomic absorption spectrometry. We measured the concentration of Mn (ig/ml) in the aqueous supernatant of eight PSII enriched membranes aliquots before and after $CaCl_2$ washing (see Fig. 2). To avoid any exogenous Mn (i.e. Mn not coming from PSII) controls were taken from the PSII enriched membranes before washing, not detecting any Mn in the aqueous solutions.

Protein sequence searches in public databases were done using BLAST2 [7], and HMMer [8]. Multiple sequence alignments (MSAs) were built using CLUSTAL [9] and T-coffee programs [10] and manually curated. Mature protein sequences from PsbO, PsbP and PsbQ from 10 organisms were selected and aligned. The organisms were: 5 higher plants—*Spinacia oleracea* (SPIOL), *Arabidopsis thaliana* (ARATH), *Pisum sativum* (PISSA), *Nicotiana tabacum* (NICTA), *Lycopersicon esculentum* (LYCES); 2 green algae—*Chlamydomonas reinhardtii* (CHLRE), *Bigelowiella natans* (BIGNA); and 3 cyanobacteria—*Synechocystis* sp. *PCC 6803* (SYNY3); *Nostoc* sp. *PCC 7120* (ANASP); *Thermosynechococcus elongatus BP-1* (THEEL). For the case PsbR only higher plant homologous sequences were found. Visualization and calculations on the PDB files were performed using Swiss-PDBViewer [11]. Search for structural classification was done on SCOP [12].

3. Results

3.1. Roles of the 4 extrinsic proteins associated to the plant OE complex

PsbO (33 kDa), PsbP (23 kDa), PsbQ (17 kDa), PsbR (10 kDa) are four extrinsic proteins (OEEps) associated to the lumenal side of PSII in higher plants [13]. They play an important role in maintaining oxygen evolution activity at physiological rates, but still many unclear points and questions are in the scientific arena about their specific biomolecular function and activity. PsbO is ubiquitously present in all known oxyphototrophs; however, PsbP, PsbQ and PsbR are only found in higher plants, since they are not present in known oxyphotosynthetic prokaryotes or in non-green algae (i.e. red algae). Cyanobacteria and red algae include extrinsic proteins PsbV and PsbU, but do not have the three higher plant specific OEEps, rather some of them only have some distant relatives to PsbP and PsbQ (see for details [14]). As far as we know PsbR is neither present in green algae. In this paper we address the question of why plants needed to "invent" these proteins (PsbP, PsbQ and PsbR) associated to the OE complex. To tackle this question we have done a comparative analysis of known functional data, sequences, structures and evolutionary information.

Table 1 presents a summary of the structural data and main functions attributed according to the literature to the four OEEps from plants: PsbO, P, Q and R. In terms of their structural characteristics, the table includes the PDBs of the structures of plant PsbP and PsbQ: *N. tabacum* PsbP at 1.60 Å (1V2B) [15] and *S. oleracea* PsbQ at 1.49 Å (1VYK) [16] and at 1.95 Å (1NZE) [17]; and describes the domains architecture, the SCOP structural classification and the Pfam multiple sequence alignment (MSA) and profiling of these proteins. For PsbO, the PDB is the one corresponding to cyanobacterial PSII (1S5L) [1], because the structure of this protein is at present only known as attached to PSII for prokaryotes, though it is expected to be quite similar in plants. The architecture of this protein has been analysed in detail in [18]. Finally, in the case of PsbR, there are not 3D structural data and the description of 2 domains comes from the prediction using a MSA of PsbR family and its Pfam file quoted in the table.

Table 1
Summary of the structural data and main functions attributed at present to the four OEEps from plants: PsbO, PsbP, PsbQ and PsbR

PsbO (33 kDa, 248 aa) 2 domains: 1st β-barrel cylinder, 2nd α-helix + loops head (within PSII pdb 1S5L, Pfam PF01716)
- essential for fully operational OEC since it is present in all known oxyphototrophs [13]
- stabilizes the Mn_4Ca cluster providing some ligands [1,2]
- conformational sensitive to Ca^{2+} and pH (active bound state = $+Ca^{2+}$ and pH ≤ 6.5) [5,30]
- seems to bind GTP/GDP, so may be involved in GTPase activity [25]

PsbP (23 kDa, 186 aa) 1 domain: 3 layer (αβα)-sandwich (pdb 1V2B, Pfam PF01789, SCOP Superfamily 55724:Mog1p/PsbP-like)
- essential for fully operational OEC *in vivo* (according to ΔpsbP in *N. tabacum*) [19]
- involved in Ca^{2+} binding and supply, increasing the OEC affinity for Ca^{2+} and Cl^- [21]
- may bind Mn^{2+} acting as a reservoir to keep/deliver Mn [22] (this binding is not in the crystal)
- may be a GTPase binding-interacting protein (according to homology with 1EQ6) [15]
- closely associated to PsbR and PsbJ [23]
- binds to PsbO head-domain by a positively charged Lys-rich region [14,26]

PsbQ (16 kDa, 149 aa) 2 domains: 1st Nt PPII, β-strands, loops, 2nd Ct 4α-helix up-down bundle (pdb 1VYK and 1NZE, Pfam PF05757, SCOP Superfamily 101113:OEEp3)
- required for PSII stability under low light conditions (according to ΔpsbQ in *A. thaliana*) [20]
- facilitates protein–protein interaction (regions: N-t polyP helix, Lys-rich face, ...) [16,27]
- binds to PsbO external cylinder-domain by a positively charged Lys-rich region [14,27]

PsbR (10 kDa, 99 aa) may be 2 domains: 1st short intrinsic, 2nd extrinsic (unknown structure, Pfam PF04725)
- closely associated to PsbP and PsbJ [23]
- associated to the OEC since it falls apart from PSII only after all 4 Mn are lost [this paper]

The functional information attributed to the OEEps from plants is also presented in Table 1 including references. Important information comes from the recent studies done on transgenic plants where PsbP [19] or PsbQ [20] proteins are not present. In *N. tabacum* lacking PsbP, PSII was hypersensitive to light and rapidly inactivated when the repair process of damaged PSII was inhibited. Moreover, the manganese cluster of PsbP-deficient leaves was markedly unstable. However, PsbQ-deficient *N. tabacum* plants did not show phenotypic alterations, suggesting that PsbQ is dispensable but PsbP is essential for full PSII function under normal growth conditions [19]. A more recent study on *A. thaliana* where both PsbQ genes (*psbQ-1* and *psbQ-2*) have been suppressed, confirms no phenotypic alteration under normal growth light but shows that PsbQ is needed for photoautotrophy under low light conditions [20]. PsbP has been implicated in Ca^{2+} binding and supply, increasing the affinity of the OE center for Ca^{2+} and Cl^- [21]. However, more recently spinach PsbP has been reported to bind Mn^{2+} and suggested to act as a reservoir capable of binding and delivering manganese to the OE center [22]. Other recent

Table 2a
Relative conservation of PsbO, PsbP and PsbQ along the MSAs that include the sequences from 5 higher plants, 2 green algae and 3 cyanobacteria (the 10 species are quoted in Methods)

PsbO, PsbP, PsbQ: relative conservation along higher plants (hp), green algae (ga) and cyanobacteria (cyano) for the same 10 species										
	10sq (5 hp+2 ga+3 cyano)			7sq (5 hp+2 ga)			5sq (5 hp)			5 higher plants vs. 2 green algae
	ali length	aa ID	% ID	ali length	aa ID	% ID	ali length	aa ID	%ID	hp distinct regions that are not in ga
PsbO	266	66	24.81%	253	123	48.62%	250	193	77.20%	(1) Nt starting motif EGAPKR (2) Region between 2 conserved Cys (3) Motif GGRGDEE
PsbP	193	13	6.74%	189	65	34.39%	186	125	67.20%	Conservation >30% plants vs. algae (1) Two *EGG* motifs conserved (2) H4 GDKRWFKG motif conserved
PsbQ	169	2	1.18%	156	22	14.10%	149	78	52.35%	Conservation <15% plants vs. algae (1) Nt polyP-rich region (2) H2 and H4 two Y motifs

studies on transgenic PsbR-deficient *A. thaliana* (ΔpsbR) have shown that PsbP and PsbR are direct assembly partners, while PsbQ is not so closely related to PsbR [23]. With respect to PsbO, our detailed structural analyses indicated that most probably PsbP binds to PsbO closer to its headdomain, and PsbQ binds to PsbO closer to its external cylinder-domain [14]. This suggestion is supported by the structural reconstruction of the lumenal extrinsic side of higher plants PSII using low resolution 3D EM map of PSII and 3D structures of PsbP and PsbQ [3]. This modelling also indicates that PsbP protein not only interacts with the PsbO but also with the lumenal surface of the PSII core in the vicinity of CP43. Cross-reconstitution of the extrinsic proteins and PSII from *C. reinhardtii* and *S. oleracea* indicated that in the green algal PsbP and Q bound to PSII independent of the other OEEps, but this was not the case in the higher plant; and higher plant PsbQ only bound specifically to higher plant PsbO [24].

A first conclusion from all these results is that they do not support the concept that the PsbP and PsbQ are structural and functional replacements for the cyanobacterial PsbU and PsbV in PSII of plants; but a second more challenging conclusion from the data is the suggestion of different roles for PsbP and PsbQ in PSII of plants, including the likelihood that PsbP is in closer association to the OE catalytic center and also with closer links to PsbR (see Table 1).

The proposal of specific roles for the OEEps in eukaryotic higher plant thylakoids has been pushed and enhanced by the interesting experiments that demonstrated that PsbO from plants can bind GTP/GDP, and so it may be involved in GTPase activity [25]. Structural analysis found PsbP to be homologous to Mog1p (1EQ6) (first reported in Ifuku et al. [15], also indicated in [14]). Mog1p is a yeast regulatory protein involved in interaction with a GTPase; and this underlines the indication of new functional playgrounds for the OEEps. Moreover, the detection of GTP/GDP in the thylakoid lumen opens new metabolic and regulatory perspectives to the proteins working in this chloroplast compartment, expanding its significance beyond a pure bioenergetic function [25].

3.2. Comparison of PsbO, PsbQ, PsbP from 10 common organisms.

Evolutionary studies have been carried out using sequences and sequence profiles for each one of the 3 major OEEps: PsbO, PsbP and PsbQ [13,14]. These studies provide means of tracing the phylogenetic tracks that each family has followed. However, to find and clarify better the differences between these 3 major OEEps within plants and green algae to those of prokaryotes, a new approach is to compare the sequence homology of these 3 proteins in an identical set of organisms including representatives of cyanobacteria, green algae and higher plants. This has been done and Tables 2a and 2b present the data coming from 3 multiple sequence alignments (MSAs) built using 10, 7 and 5 sequences corresponding to 5 higher plant proteins, 2 green algae proteins and 3 cyanobacterial proteins. Each one of these 3 MSAs was built for PsbO, PsbP and PsbQ, obtaining a set of 3 × 3 MSAs. For the study it was necessary to have the sequences of the 3 proteins in the same organisms (or homologous proteins for the case of PsbP-like and PsbQ-like from cyanobacteria). Sequences were found only for 10 species, as indicated in Methods. The resulting analyses of relative conservation in higher plants, green algae and cyanobacteria, presented in Table 2a, show that PsbO is conserved with about 24% identity along all these species; but the PsbP-like and PsbQ-like proteins from cyanobacteria are not closely related, at least by sequence, with the plant PsbP and PsbQ proteins, since the relative conservation

Table 2b
Schematic data about of the presence of PsbP homologous proteins and PsbQ homologous proteins within the main phylogenetic branches of oxyphototrophic organisms

PsbP and PsbQ like proteins present along phylogenetic main branches						
	Ancient cyanobacteria	Cyanobacteria	Green oxyphotobacteria	non-green algae	green algae (ga)	Higher plants (hp)
P homolog type	cyanoP	cyanoP	cyanoP	–	ga PsbP	hp PsbP
Q homolog type	–	cyanoQ	–	PsbQ′	ga PsbQ	hp Psb0

drops to 6% and 1%. By contrast, the conservation between the higher plans is very clear with relative identities of 77% for PsbO, 67% for PsbP and 52% for PsbQ. These figures also suggest that in all the plants studied these 3 OEEps will have the same structure and the same function, with PsbO being the most conserved and PsbQ having the lowest conservation pressure

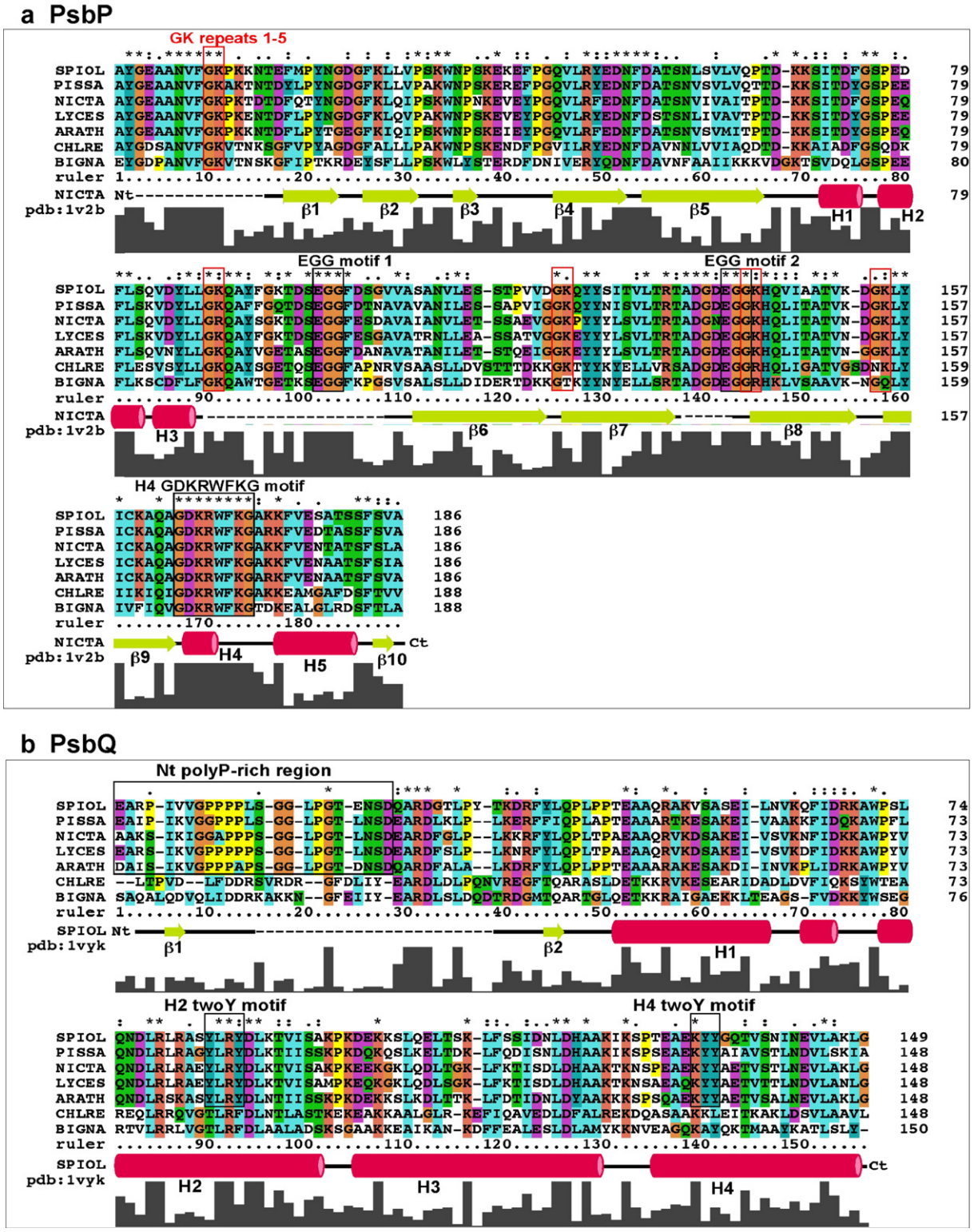

Fig. 1. MSAs of PsbP and PsbQ from 7 species (5 higher plants and 2 green algae quoted in Methods). The alignments include the known secondary structure for *Nicotiana tabacum* PsbP (pdb 1V2B) and *Spinacea oleracea* PsbQ (pdb 1VYK). Adjusted to the sequence space, the alpha-helices are drawn as purple cylinders and the beta-strands as yellow arrows. The colour code of the residues in the alignments corresponds to the standard provided by CLUSTAL. Some significant motifs are marked with boxes and labeled.

within its sequence since it changes 48% within 5 plants. Finally, the comparison of green algae and higher plant proteins shows a clear difference between PsbP and PsbQ; indicating that most probably PsbP is structurally and functionally conserved (relative identity higher than 30%), but PsbQ from plants is in some way different to PsbQ from algae (relative identity bellow 15%). Evolutionary phylogenetic analysis of these two proteins [13,14] showed that PsbP-like proteins are present in more ancient oxyphototrophs and PsbQ-like proteins may appear later in evolution. These observations are presented schematically in Table 2b.

3.3. Structural comparison of PsbP and PsbQ in green algae and plants

To explore the sequence and structure convergence of the PsbP and PsbQ protein families in green algae versus higher plants in more depth, two MSAs of the 7 species quoted above are presented in Fig. 1. The alignments also integrate the secondary structure information coming from the 3D known structures of *N. tabacum* PsbP (pdb 1V2B) and *S. oleracea* PsbQ (pdb 1VYK). For the case of PsbP (Fig. 1a) the data show a good conservation along all the sequences, therefore allowing the identification of short conserved linear motifs that are present in algae and in plants: two EGG motifs; a GDKRWFKG motif located around alpha-helix 4; a GK repeat found five times. By contrast, in the PsbQ MSA (Fig. 1b) the conservation profile is very much diminished and a clear difference can be observed between green algae and higher plants, which is most obvious within the N-terminal region. In this region PsbQ from plants have the polyproline motif that corresponds in 3D to a very peculiar left-handed helix PPII type [16], that can be related to protein–protein interaction. Also two short linear motifs that include two conserved tyrosine residues (Y) within the alpha-helices 2 and 4, are found only in the higher plant sequences.

3.4. New data about the role of PsbR with respect to the manganese cluster

Not a great deal of data have been published about the specific function of the smallest of the plant OEEps that is PsbR. To find some new information about this protein, PSII enriched membranes were isolated and after checking the correct PSII activity (by oxygen evolution and fluorescence measurements) they were washed using 1M $CaCl_2$ at pH 6.0. Just before and after the washing aliquots were taken to measure the manganese concentration in the supernatant of the membranes and the proteins in the membranes. Several SDS/PAGE analyses were performed to quantified the presence of proteins PsbO, P, Q and R before and after the washing in the PSII membranes (in Fig. 2b, lane 0 marks the OEEps). Multiple densitometry analyses in several ran gels indicated that the amount of PsbO, P, Q and R in the PSII enriched membranes was normal before the washing. After the washing, PsbO, P and Q bands disappear detecting less than 5% signal for these bands (see Fig. 2b, lanes 1–8). A faint band above PsbO can be seen in the lanes but it is not the 33 kDa OEEp. The PsbR band remains present with minimal

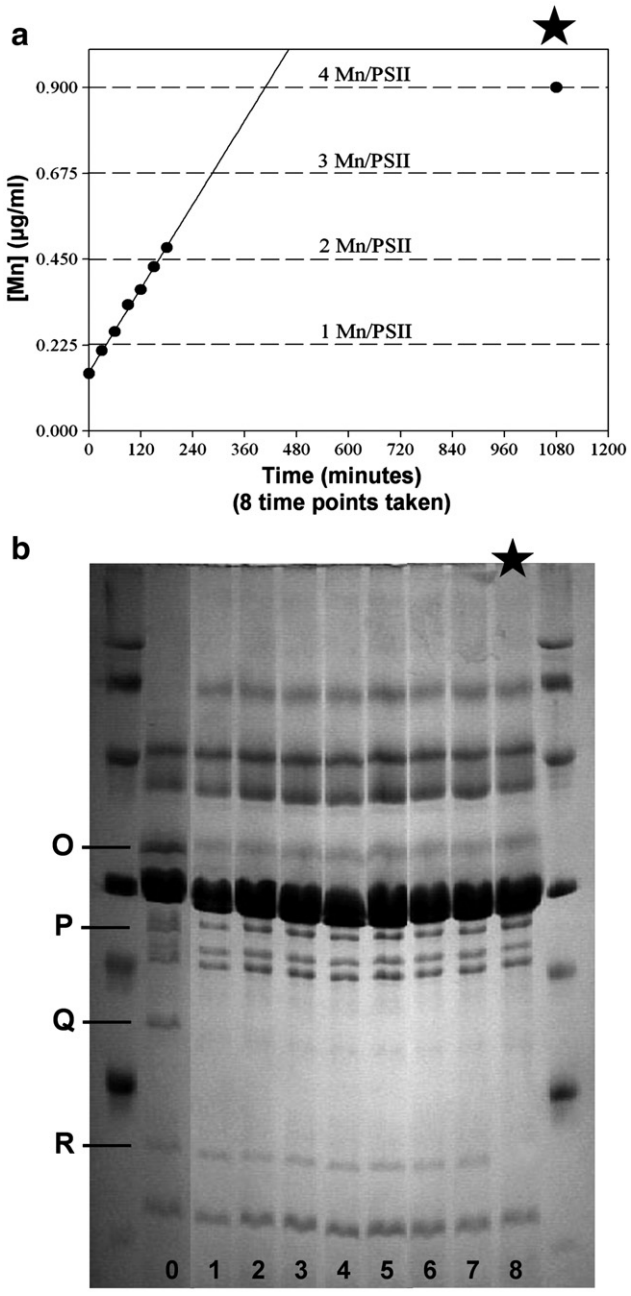

Fig. 2. (a) Measurement of the amount of manganese per reaction center present at 8 consecutive time points in the supernatant of the PSII enriched membranes after washing with 1 M $CaCl_2$ at pH 6.0. (b) Separation and detection of proteins by SDS/PAGE from the PSII-enriched membrane samples before washing (time 0) and after washing at the same aliquots of the 8 time points indicated above. The location of the OEEps in the protein gels is indicated.

alteration (i.e. signal >90%) till lane 7, but in lane 8 this band also disappears. The comparison of data in Fig. 2a and b reveals that after the $CaCl_2$ washing the manganese atoms (4 Mn per reaction center) start to drop out, but PsbR remains bound to the PSII membranes till all the manganeses are lost. The observed correspondence between the figures of Mn present at each time point (Fig. 2a) and the PsbR present in the protein PAGE gel for the same time course aliquots (Fig. 2b) gives evidence to an association between PsbR and the Mn cluster. This observation gives new support to the location of

PsbR in the vicinity of the OE center, and, so, closer to PsbP, as indicated above.

4. Discussion

4.1. Model location and arrange of the OEEps in PSII from plants

The results presented in this study combined to many of the evidences quoted from the literature allows us to suggest a model about the location of the 4 OEEps in the lumenal side of PSII complex in plants. Fig. 3a shows a drawing depicting a schematic arrangement of PsbO, PsbP, PsbQ and PsbR within PSII extrinsic region. PsbO, in algae and plants, is located closest to the OE Mn catalytic center with an elongated shape coming towards the lumen. This is based on structural analysis using PSII 3D data, considering PsbO from plants to be very similar to cyanobacterial PsbO and conserved along evolution. However, the figures (see Fig. 3a and b) propose two main differences between green algae and higher plants: (i) a closer

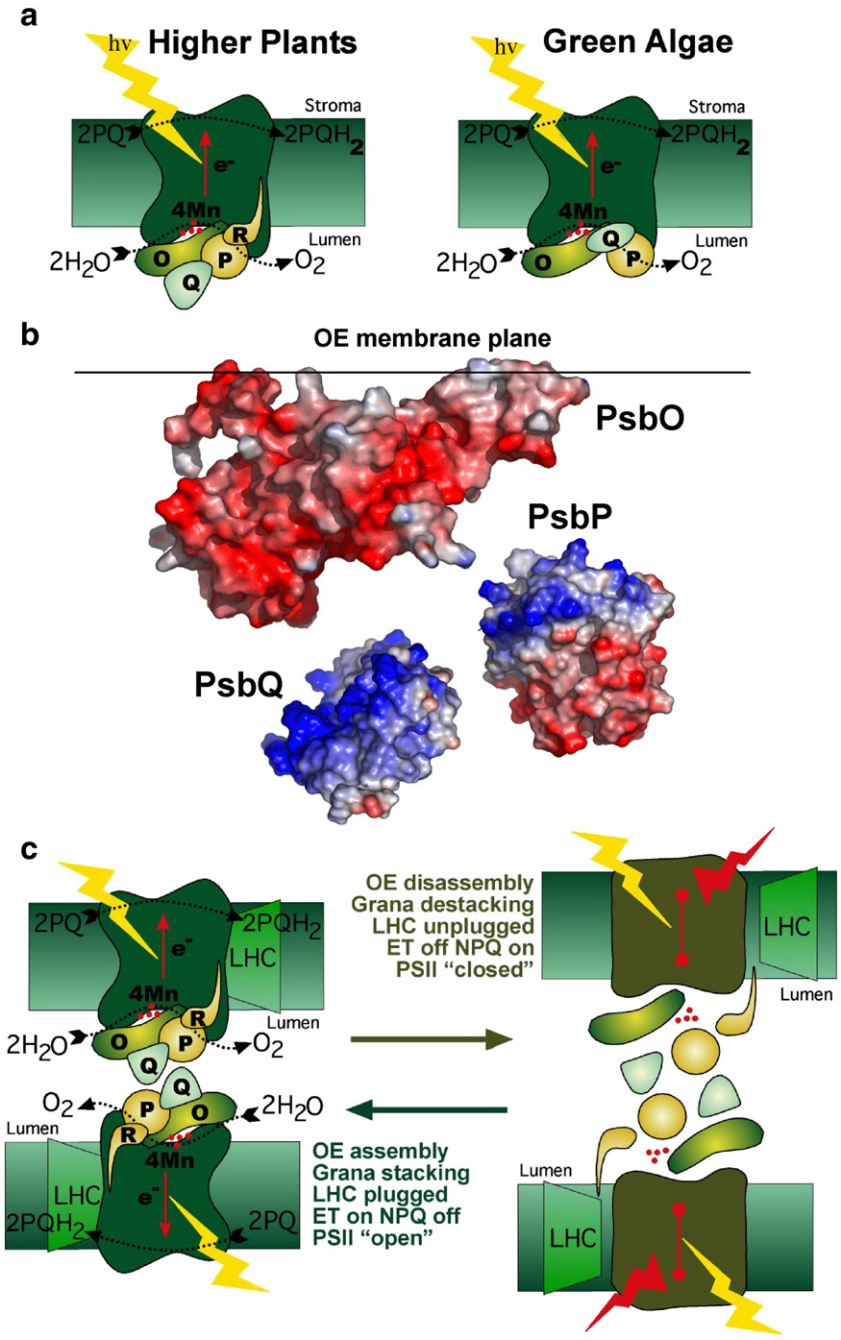

Fig. 3. (a) Schematic drawing of PSII showing the proposed arrangement of PsbO, PsbP, PsbQ and PsbR in the extrinsic lumenal region. (b) 3D surface models of PsbO, PsbP and PsbQ, built using the respective PDBs quoted in Table 1. (c) Schematic drawing showing a proposed role for the OEEps in the correct 3D assembly of the grana lamellae and in PSII full activity.

interaction between PsbQ and PsbP in plants; (ii) a larger external interacting N-terminal domain present only in PsbQ from plants. The structural analysis of 3D data from crystallized PsbO, P and Q and a low resolution model of higher plant PSII derived from electron microscopy [3] also indicated that PsbP interaction interface is most probably closer to the head domain of PsbO and PsbQ interaction interface is closer to the cylinder external domain of PsbO [14]. This is drawn graphically in Fig. 3b. Another important difference reported by Suzuki et al. [24] is that green algal PsbP and Q bound to PSII independent of the other OEEps, but this was not the case in higher plant. We try to reflect this in Fig. 3a locating PsbP and Q in green algae in places that will allow an independent binding to PSII. Finally, studies on the structural association of PsbP [26] and PsbQ [27] with PsbO and PSII in higher plants have revealed that the domains most probably involved in such interactions are in both cases positively charged regions. The location of the OEEps in Fig. 3b is congruent with these reports.

4.2. Hypothesis proposal: role of higher plant OEEps in grana dynamics

In this last section we propose a hypothesis for a possible function of the OEEps in the correct 3D assembly of the grana lamellae needed for higher plant PSII full activity and regulation; including a particular role for PsbQ in the protein–protein interactions needed for such assembly. This is a working hypothesis presented as an integrative approach to better understand the dynamic function of PSII in higher plant thylakoids. A model for this hypothesis is drawn in Fig. 3c, suggesting some relationship between the higher plant "PSII OEEps" and the higher plant "PSII functional state". In this way, OE assembly, grana stacking, light harvesting complexes (LHC) plugging, PSII linear electron transfer (ET), and PSII reaction centers opening will occur only when the OEEps are fully attached to PSII. In fact, PsbP and PsbQ have been found to associate with PSII only in the grana thylakoids [28]. By contrast, OE disassembly, grana destacking, LHC unplugging, PSII linear ET interruption, non-photochemical quenching (NPQ) increase, and PSII reaction centers closing are conditions in which PsbQ, PsbP, PsbR, and may be PsbO will be most probably detached from PSII. Recent studies using transgenic plants defective in PsbP [19] or PsbQ [20] are showing that both proteins are needed for PSII stability in higher plants. These studies show that ΔPsbP tobacco plants were hypersensitive to light [19] and that ΔPsbQ *Arabidopsis* plants were unstable under low light conditions [20]. Therefore, in both cases it seems that PSII normal activity is not obliterated, but the regulation and full dynamics of PSII in higher plant thylakoids is clearly dependent on the presence of these proteins. Finally, the suggestion that the most external part of PSII supercomplex may interact in the grana lumen with an opposite PSII supercomplex is supported by the fact that higher plant PsbQ has been reported to include several structural domains very suitable to facilitate protein–protein interactions [16,27] and that the recent higher plant PSII 3D structural model places PsbQ in the most external part of the OE extrinsic region [3].

Although grana are not essential for photosynthesis, they are ubiquitous in higher plants [29]. Therefore, grana may have been selected during evolution for the functional advantages that they confer on higher plants [29]. The biomolecular elements directly involved in grana stacking are not known at present, but it is clear that they will be in some way exclusive to higher plants. It is clear that the hypothetical involvement of higher plant OEEps in grana dynamics has to be specifically proven, but our proposal opens new venues for experimental trials to find out which are the proteins involved in the macromolecular structure and dynamics of grana and stroma lamellae, and in reversible thylakoid membrane stacking.

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